TREHALOSE SYNTHASE PROTEIN, GENE, PLASMIDS, MICROORGANISMS, AND A PROCESS FOR PRODUCING TREHALOSE

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BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a trehalose-producing microorganism and a process for producing trehalose. It also relates to a novel trehalose synthase protein, a trehalose synthase gene, recombinant plasmids carrying said trehalose synthase gene, and transformed microorganisms with said recombinant plasmids.

DESCRIPTION OF THE PRIOR ART

Trehalose is a non-reducing disaccharide, two saccharides of which are linked by α-1,1 bond: α-D-glucopyranosyl-α-D-glucopyranoside. It has wide application in medicines, foods, and cosmetics. However, its utilization has been greatly restricted because its production to date has been inefficient and expensive.

Japanese Laid-open Patent Nos. Hei5-91890 and Hei6-145186 disclose methods for extracting trehalose from yeasts. There are several methods for isolating trehalose from fermented microorganism cultures, such as *Arthrobacter* (T. Suzuki, Agric. Biol. Chem., 33(2), 1969), *Nocardia* (Japanese Laid-open Patent No. Sho50-154485), *Micrococcus* (Japanese Laid-open Patent No. Hei6-319578), amino acid-fermenting yeast, *Brevibacterium* (Japanese Laid-open Patent No. Hei5-211882), and yeast (Yoshikwa, etc., Biosci. Biotech. Biochem., 1994, 58, 1226-12300). Additionally, a method for producing trehalose by using recombinant plants including bacterial genes capable of converting glucose into trehalose is described in M. Scher, Food Processing, April, 95-96, 1993. Japanese Laid-open Patent No. 83-216695 discloses a method for converting maltose into trehalose by using maltose phosphorylase and trehalose phosphorylase. However, these methods are not effective, because their procedures are complicated and their yields are low.

Several enzymatic methods have been published recently. Japanese Laid-open Patent No. Hei7-143876 and EPO 628630 A2 discloses a two-step enzymatic conversion method in which starch is converted into trehalose by maltooligosyl trehalose synthase and maltooligosyl trehalose trehalohydrolase. Japanses Laid-open Patent No. Hei7-170977 and Korean Laid-open Patent No. 95-3444 disclose one-step enzymatic conversion methods in which maltose is directly converted into trehalose by trehalose synthase. However, there is still a need to increase the titer of the trehalose synthase enzyme so that production of trehalose from maltose becomes more efficient in yield and cost.

We have invested much effort over the last several years in isolating microorganisms able to convert maltose into trehalose from soil. We have successfully screened a novel strain which highly expresses trehalose and, unexpectedly, generates no byproducts, unlike all known microorganisms. Its morphological and physiological characteristics identify it as a novel *Pseudomonas stutzeri* strain. This strain has been designated as *Pseudomonas stutzeri* CJ38.

We isolated a trehalose synthase gene from chromosomes of *Pseudomonas stutzeri* CJ38 and determined its nucleotide sequence by cloning it into known vector pUC18 with restriction enzyme *Sau*3AI. In addition, we isolated a trehalose synthase protein from *Pseudomonas stutzeri* CJ38 and determined its amino acid sequence using standard methods. It was found that these sequences are apparently different from the sequences of the trehalose synthase gene and all proteins known hitherto. This invention was achieved by constructing recombinant plasmids carrying the trehalose synthase gene so that the trehalose synthase enzyme encoded in said gene can be expressed in large amounts.

SUMMARY OF THE INVENTION

The present invention provides a novel microorganism, *Pseudomonas stutzeri* CJ38, that produces trehalose from maltose. This strain was deposited at the Korea Culture Center of Microorganisms. Seoul, Korea, as the accession number KCCM 10150 on February 12, 1999 under the Budapest Treaty. This strain is very valuable

as it does not generate byproducts such as glucose when converts maltose into trehalose.

The present invention also provides a novel trehalose synthase protein with the following amino acid sequence:

Met Ser Ile Pro Asp Asn Thr Tyr Ile Glu Trp Leu Val Ser Gln
5 10 15
Ser Met Leu His Ala Ala Arg Glu Arg Ser Arg His Tyr Ala Gly
20 25 30
Gln Ala Arg Leu Trp Gln Arg Pro Try Ala Gln Ala Arg Pro Arg
35 40 45
Asp Ala Ser Ala Ile Ala Ser Val Trp Phe Thr Ala Tyr Pro Ala
50 55 60
Ala Ile Ile Thr Pro Glu Gly Gly Thr Val Leu Glu Ala Leu Gly
65 70 75
Asp Asp Arg Leu Trp Ser Ala Leu Ser Glu Leu Gly Val Gln Gly
80 85 90
Ile His Asn Gly Pro Met Lys Arg Ser Gly Gly Leu Arg Gly Arg
95 100 105
Glu Phe Thr Pro Thr Ile Asp Gly Asn Phe Asp Arg Ile Ser Phe
110 115 120
Asp Ile Asp Pro Ser Leu Gly Thr Glu Glu Gln Met Leu Gln Leu
125 130 135
Ser Arg Val Ala Ala Ala His Asn Ala Ile Val Ile Asp Asp Ile
140 145 150
Val Pro Ala His Thr Gly Lys Gly Ala Asp Phe Arg Leu Ala Glu
155 160 165
Met Ala Tyr Gly Asp Tyr Pro Gly Leu Tyr His Met Val Glu Ile
170 175 180
Arg Glu Glu Asp Trp Glu Leu Leu Pro Glu Val Pro Ala Gly Arg
185 190 195
Asp Ser Val Asn Leu Leu Pro Pro Val Val Asp Arg Leu Lys Glu
200 205 210

Lys His Tyr Ile Val Gly Gln Leu Gln Arg Val Ile Phe Phe Glu
215 220 225
Pro Gly Ile Lys Asp Thr Asp Trp Ser Val Thr Gly Glu Val Thr
230 235 240
Gly Val Asp Gly Lys Val Arg Arg Trp Val Tyr Leu His Tyr Phe
245 250 255
Lys Glu Gly Gln Pro Ser Leu Asn Trp Leu Asp Pro Thr Phe Ala
260 265 270
Ala Gln Gln Leu Ile Ile Gly Asp Ala Leu His Ala Ile Asp Val
275 280 285
Thr Gly Ala Arg Val Leu Arg Leu Asp Ala Asn Gly Phe Leu Gly
290 295 300
Val Glu Arg Arg Ala Glu Gly Thr Ala Trp Ser Glu Gly His Pro
305 310 315
Leu Ser Val Thr Gly Asn Gln Leu Leu Ala Gly Ala Ile Arg Lys
320 325 330
Ala Gly Gly Phe Ser Phe Gln Glu Leu Asn Leu Thr Ile Asp Asp
335 340 345
Ile Ala Ala Met Ser His Gly Gly Ala Asp Leu Ser Tyr Asp Phe
350 355 360
Ile Thr Arg Pro Ala Tyr His His Ala Leu Leu Thr Gly Asp Thr
365 370 375
Glu Phe Leu Arg Met Met Leu Arg Glu Val His Ala Phe Gly Ile
380 385 390
Asp Pro Ala Ser Leu Ile His Ala Leu Gln Asn His Asp Glu Leu
395 400 405
Thr Leu Glu Leu Val His Phe Trp Thr Leu His Ala Tyr Asp His
410 415 420
Tyr His Tyr Lys Gly Gln Thr Leu Pro Gly Gly His Leu Arg Glu
425 430 435
His Ile Arg Glu Glu Met Tyr Glu Arg Leu Thr Gly Glu His Ala
440 445 450

Pro Tyr Asn Leu Lys Phe Val Thr Asn Gly Val Ser Cys Thr Thr
455 460 465
Ala Ser Val Ile Ala Ala Ala Leu Asn Ile Arg Asp Leu Asp Ala
470 475 480
Ile Gly Pro Ala Glu Val Glu Gln Ile Gln Arg Leu His Ile Leu
485 490 495
Leu Val Met Phe Asn Ala Met Gln Pro Gly Val Phe Ala Leu Ser
500 505 510
Gly Trp Asp Leu Val Gly Ala Leu Pro Leu Ala Pro Glu Gln Val
515 520 525
Glu His Leu Met Gly Asp Gly Asp Thr Arg Trp Ile Asn Arg Gly
530 535 540
Gly Tyr Asp Leu Ala Asp Leu Ala Pro Glu Ala Ser Val Ser Ala
545 550 555
Glu Gly Leu Pro Lys Ala Arg Ser Leu Tyr Gly Ser Leu Ala Glu
560 565 570
Gln Leu Gln Arg Pro Gly Ser Phe Ala Cys Gln Leu Lys Arg Ile
575 580 585
Leu Ser Val Arg Gln Ala Tyr Asp Ile Ala Ala Ser Lys Gln Ile
590 595 600
Leu Ile Pro Asp Val Gln Ala Pro Gly Leu Leu Val Met Val His
605 610 615
Glu Leu Pro Ala Gly Lys Gly Val Gln Leu Thr Ala Leu Asn Phe
620 625 630
Ser Ala Glu Pro Val Ser Glu Thr Ile Cys Leu Pro Gly Val Ala
635 640 645
Pro Gly Pro Val Val Asp Ile Ile His Glu Ser Val Glu Gly Asp
650 655 660
Leu Thr Asp Asn Cys Glu Leu Gln Ile Asn Leu Asp Pro Tyr Glu
665 670 675
Gly Leu Ala Leu Arg Val Val Ser Ala Ala Pro Pro Val Ile
680 685

In addition, the present invention provides a novel trehalose synthase gene with the following nucleotide sequence:

GATCGCTGGC GTACTGCAGG TAGAGCAGGC GCATCGGCCC CCAGGGCGCA TCGGCCGGCT 60

CCGCTGTGCC CTGCTGGTTC ATGAAGCGGA CGAAGCGGCC ATCGCGGAAC CGTGGACGCC 120

ATTCGGGGCT GTCCGGGTCG CGGCTGTCGG TGAGCGTGCG CCACAGGTCG CTGCGAAACG 180

GCGGACCGCT CCAAAGCGCG CCGTGGATGG GATCGCCGAG CAGTTCGTGC AGCTCCCAGG 240

AACGTTGCGA ATGCAGCGCG CCGAGGCTCA GGCCATGCAG ATACAGGCGC GGTCGGCGTT 300

CGGCCGGCAG TTCGGTCCAG TAGCCATAGA TCTCGGCGAA TAGCGCGCGG GCCACGTCGC 360

GGCCGTAGTC GGCCTCCACC AGCAGCGCCA GCGGGCTGTT CAGATAGGAG TACTGCAACG 420

CCACGCTGGC GATATCGCCG TGGTGCAGGT ATTCCACTGC GTTCATCGCC GCCGGGTCGA 480

TCCAGCCGGT ACCGGTGGGC GTCACCAGCA CCAGCACCGA TCGCTCGAAG GCGCCGCTGC 540

GCTGCAGCTC GCGCAAGGCC AGACGCGCCC GCTGGCGCGG GGTCTCTGCC GCGCGCAGAC 600

CGACGTAGAC GCGAATCGGC TCGAGCGCCG AGCGGCCGCT CAAGACGCTG ATATCCGCCG 660

CCGACGGGCC GGAGCCGATG AACTCGCGGC CGGTGCGGCC CAGCTCCTCC CAGCGCAGCA 720

ACGAGGCCCG GCTGCCGCTT TTCAGCGGCG AGGCCGGTGG CGCCGTCTCC GGTTCGATCA 780

GGGCGTCGTA CTGCGCGAAG GATGCGTCCA GCATGCGCAG TGCCCGCGCC GCCAGCACAT 840

CGCTGAGCAG CGACCAGAAC AGCGCCAGCG CCACCAGCAC GCCGATCACG TTGGCCAGGC 900

GCCGTGGCAG CACGCGGTCG GCGTGCCGCG AGACGAAGCG CGACACCAGC CGATACAGAC 960

GCGCCAGCGT CAGCAGGATG AGAAAGGTCG CCAGCGCGGT GAGAATGACT TCGAGCAGGT 1020

GCGCACTGCT CACCGGCGGC ATGCCCATCA GCGCGCGTAC CGCGTTCTGC CAGCCGGCGA 1080

CCTGGCTGAG GAAATACCCG GCCAGCAGCA GGCAGCCGAC CGCGATCAGC AGATTGACCC 1140

GCTCGCGCTG CCAGCCTGGG CGCTCCGGCA GTTCCAGATA GCGCCACAGC CAGCGCCAGA 1200

ACACGCCGAG GCCATAGCCC ACCGCCAGCG CCGCGCCGGC CAGCACGCCC TGGCTCAGCG 1260

TCGAGCGCGG CAGCAGCGAT GGCGTCAGCG CCGCGCAGAA GAACAGCGTG CCCAGCAGCA 1320

GGCCGAAACC GGACAGCGAG CGCCAGATAT AGAGGACGGG CAGGTGCAGC ATGAAGATCT 1380

CCGCGGTCGG GTGACGGCGT CGCGCCTCGG CATATCGAGG CGTGTCCGGT CGTGCGGTTC 1440

CCGTGATGGT CCGCAGCAGG CCAATCCGAT GCAACGATGG CCGAGCGGCC GACTCAAACG 1500

TCTACATTTC CCTAGTGCTG CCGGAACCGA TCGCCG 1536

ATG AGC ATC CCA GAC AAC ACC TAT ATC GAA TGG CTG GTC AGC CAG TCC 1584

ATG CTG CAT GCG GCC CGC GAG CGG TCG CGT CAT TAC GCC GGC CAG GCG 1632

CGT CTC TGG CAG CGG CCT TAT GCC CAG GCC CGC CCG CGC GAT GCC AGC 1680

GCC ATC GCC TCG GTG TGG TTC ACC GCC TAT CCG GCG GCC ATC ATC ACG 1728

CCG GAA GGC GGC ACG GTA CTC GAG GCC CTC GGC GAC GAC CGC CTC TGG 1776

AGT GCG CTC TCC GAA CTC GGC GTG CAG GGC ATC CAC AAC GGG CCG ATG 1824
AAG CGT TCC GGT GGC CTG CGC GGA CGC GAG TTC ACC CCG ACC ATC GAC 1872
GGC AAC TTC GAC CGC ATC AGC TTC GAT ATC GAC CCG AGC CTG GGG ACC 1920
GAG GAG CAG ATG CTG CAG CTC AGC CGG GTG GCC GCG GCG CAC AAC GCC 1968
ATC GTC ATC GAC GAC ATC GTG CCG GCA CAC ACC GGC AAG GGT GCC GAC 2016
TTC CGC CTC GCG GAA ATG GCC TAT GGC GAC TAC CCC GGG CTG TAC CAC 2064
ATG GTG GAA ATC CGC GAG GAG GAC TGG GAG CTG CTG CCC GAG GTG CCG 2112
GCC GGG CGT GAT TCG GTC AAC CTG CTG CCG CCG GTG GTC GAC CGG CTC 2160
AAG GAA AAG CAC TAC ATC GTC GGC CAG CTG CAG CGG GTG ATC TTC TTC 2208
GAG CCG GGC ATC AAG GAC ACC GAC TGG AGC GTC ACC GGC GAG GTC ACC 2256
GGG GTC GAC GGC AAG GTG CGT CGC TGG GTC TAT CTG CAC TAC TTC AAG 2304
GAG GGC CAG CCG TCG CTG AAC TGG CTC GAC CCG ACC TTC GCC GCG CAG 2352
CAG CTG ATC ATC GGC GAT GCG CTG CAC GCC ATC GAC GTC ACC GGC GCC 2400
CGG GTG CTG CGC CTG GAC GCC AAC GGC TTC CTC GGC GTG GAA CGG CGC 2448
GCC GAG GGC ACG GCC TGG TCG GAG GGC CAC CCG CTG TCC GTC ACC GGC 2496
AAC CAG CTG CTC GCC GGG GCG ATC CGC AAG GCC GGC GGC TTC AGC TTC 2544
CAG GAG CTG AAC CTG ACC ATC GAT GAC ATC GCC GCC ATG TCC CAC GGC 2592
GGG GCC GAT CTG TCC TAC GAC TTC ATC ACC CGC CCG GCC TAT CAC CAT 2640
GCG TTG CTC ACC GGC GAT ACC GAA TTC CTG CGC ATG ATG CTG CGC GAA 2688
GTG CAC GCC TTC GGC ATC GAC CCG GCG TCA CTG ATC CAT GCG CTG CAG 2736
AAC CAT GAC GAG TTG ACC CTG GAG CTG GTG CAC TTC TGG ACG CTG CAC 2784
GCC TAC GAC CAT TAC CAC TAC AAG GGC CAG ACC CTG CCC GGC GGC CAC 2832
CTG CGC GAA CAT ATC CGC GAG GAA ATG TAC GAG CGG CTG ACC GGC GAA 2880
CAC GCG CCG TAC AAC CTC AAG TTC GTC ACC AAC GGG GTG TCC TGC ACC 2928
ACC GCC AGC GTG ATC GCC GCG GCG CTT AAC ATC CGT GAT CTG GAC GCC 2976
ATC GGC CCG GCC GAG GTG GAG CAG ATC CAG CGT CTG CAT ATC CTG CTG 3024
GTG ATG TTC AAT GCC ATG CAG CCC GGC GTG TTC GCC CTC TCC GGC TGG 3072
GAT CTG GTC GGC GCC CTG CCG CTG GCG CCC GAG CAG GTC GAG CAC CTG 3120
ATG GGC GAT GGC GAT ACC CGC TGG ATC AAT CGC GGC GGC TAT GAC CTC 3168
GCC GAT CTG GCG CCG GAG GCG TCG GTC TCC GCC GAA GGC CTG CCC AAG 3216
GCC CGC TCG CTG TAC GGC AGC CTG GCC GAG CAG CTG CAG CGG CCA GGC 3264
TCC TTC GCC TGC CAG CTC AAG CGC ATC CTC AGC GTG CGC CAG GCC TAC 3312
GAC ATC GCT GCC AGC AAG CAG ATC CTG ATT CCG GAT GTG CAG GCG CCG 3360

GGA CTC CTG GTG ATG GTC CAC GAG CTG CCT GCC GGC AAG GGC GTG CAG 3408
CTC ACG GCA CTG AAC TTC AGC GCC GAG CCG GTC AGC GAG ACC ATC TGC 3456
CTG CCC GGC GTG GCG CCC GGC CCG GTG GTG GAC ATC ATT CAC GAG AGT 3504
GTG GAG GGC GAC CTC ACC GAC AAC TGC GAG CTG CAG ATC AAC CTC GAC 3552
CCG TAC GAG GGG CTT GCC CTG CGT GTG GTG AGC GCC GCG CCG CCG GTG 3600
ATC TGA GCGC 3610
CCTCTTCGCG CGCCCCGGGT CCGCCGCTAT AGTGCGCAGC GCCTGGGGCG CGCATTGCCC 3670
TCGCCGTCGA GACCAGCCCG TGTCGTTCAC TTCGCTTTTC CGCCTTGCGC TGCTGCCGCT 3730
GGCGCTGCTT GCCGCACCCG TCTGGGCGCA GACCGCCTGC CCGCCCGGCC AGCAGCCGAT 3790
CTGCCTGAGC GGCAGCTGCC TCTGCGTGCC GGCCGCCGCC AGCGATCCAC AGGCGGTCTA 3850
CGACCGCGTG CAGCGTATGG CTACGCTGGC CCTGCAGAAC TGGATCCAGC AGTCGCGCGA 3910
CCGCCTGATG GCCGGCGGCG TCGAGCCGAT ACCGCTGCAC ATCCGCTCGC AGCTCGAGCC 3970
GTATTTCGAT CTTGCCGTGC TGGAGAGTGC GCGGTACCGC GTCGGCGACG AGGTGGTGCT 4030
GACTGCCGGC AACACCCTGC TGCGCAACCC GGACGTCAAT GCCGTGACCC TGATCGACGT 4090
CATCGTCTTC CGCCACGAGG AGGATGCCCG GGACAACGTC GCGCTCTGGG CCCATGAGCT 4150
CAAGCACGTC GAGCAATATC TGGACTGGGG CGTCGCCGAG TTCGCCCGGC GCTATACGCA 4210
GGATTTCCGT GCCGTGGAGC GCCCGGCCTA TGCGCTGGAG CGTGAGGTGG AAGAGGCCCT 4270
GCGCGAGACG CAGACGCGGC GCTGAGCGAG CTGATCGGTG CTGCTGCCCG CACTGGGCTG 4330
AAGCCCACCA ATGACGCCGG CGAAAACGAA AAACCCCGCC GAGGCGGGGT TTCTGACGCG 4390
GGTTGTGCGG TCAGCTCAGA ACGCCGGGAC CACGGCGCCC TTGTACTTTT CCTCGATGAA 4450
CTGGCGTACT TGCTCGCTGT GCAGCGCGGC AGCCAGTTTC TGCATGGCAT CGCTGTCCTT 4510
GTTGTCCGGA CGGGCGACCA GAATGTTCAC GTATGGCGAG TCGCTGCCCT CGATCACCAG 4570
GGCGTCCTGG GTCGGGTTCA GCTTGGCTTC CAGCGCGTAG TTGGTGTTGA TCAGCGCCAG 4630
GTCGACCTGG GTCAGCACGC GCGGCAGAGT CGCGGCTTCC AGTTCGCGGA TCTTGATCTT 4690
CTTCGGGTTC TCGGCGATGT CTTCGGCGTG GCGGTGATGC CGGCGCCGTC CTTCAGACCG 4750
ATC 4753

The present invention also provides a recombinant plasmid containing the trehalose synthase gene with the above nucleotide sequence. In a preferred embodiment, the present invention provides a recombinant plasmid pCJ104 in which the 4.7 kb *Sau*3AI DNA fragment of the trehalose synthase gene of the present invention is cloned into vector plasmid pUC18. This allow for the efficient and high

expression of the trehalose synthase gene. In a more preferred embodiment, the present invention provides a recombinant plasmid pCJ122 in which the 2.5 kb *Bam*HI-*Bgl*II DNA fragment of the trehalose synthase gene of the present invention is included in a vector plasmid pUC18, allowing for a higher expression of the trehalose synthase gene.

The present invention provides a transformed *E. coli* with a recombinant plasmid containing the trehalose synthase gene with the above nucleotide sequence. In a preferred embodiment, the present invention provides a transformed *E. coli* with a recombinant plasmid pCJ104, allowing for production of high levels of the trehalose synthase protein. In a more preferable embodiment, the present invention provides a transformed *E. coli* with the recombinant plasmid pCJ122, allowing for production of even higher levels of the trehalose synthase protein.

The present invention provides a process for producing trehalose which comprises reacting the trehalose synthase protein with the above amino acid sequence with maltose solution to obtain trehalose.

The present invention provides a process for producing trehalose which comprises crushing a transformed *E. coli* with a recombinant plasmid containing the trehalose synthase gene with the above nucleotide sequence and reacting the crushed cells with maltose solution to obtain trehalose. In a preferred embodiment, the present invention provides a process for producing trehalose which comprises crushing a transformed *E. coli* with plasmid pCJ104, centrifuging the crushed cells and reacting the resulting supernatant with maltose solution to obtain trehalose. In a more preferable embodiment, the present invention provides a process for producing trehalose which comprises crushing a transformed *E. coli* with plasmid pCJ122, centrifuging the crushed cells and reacting the resulting supernatant with maltose solution to obtain trehalose.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 shows an analysis of saccharides by thin-layer chromatography to

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which a reaction solution containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution was subjected. The symbols G. M and T indicate glucose, maltose and trehalose, respectively.

Figure 2 shows an analysis of saccharides by gas chromatography to which a reaction solution (A) containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution and a standard trehalose specimen (B) were subjected. The symbol Tre indicate trehalose.

Figure 3 shows an analysis of saccharides by high performance liquid chromatography to which a standard trehalose specimen (A), and specimens (B) and (C) were subjected. Specimen (B) was obtained just after a solution containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution was reacted completely. Specimen (C) was obtained by adding trehalase to a reaction solution containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution after completion of their reaction. The symbols Tre, Mal and Glu indicate trehalose, maltose and glucose, respectively.

Figure 4 shows a construction map of a recombinant plasmid pCJ104 including a trehalose synthase gene of the present invention.

Figure 5 shows a restriction map of a 4.7 kb *Sau*3AI fragment within a recombinant plasmid pCJ104 of the present invention.

Figure 6 shows a construction map of recombinant plasmids pCJ121 and pCJ122 of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

A microorganism which produces trehalose from maltose by trehalose synthase was isolated from soil and identified as having the morphological and physiological characteristics of *Pseudomonas stutzeri*. *Pseudomonas stutzeri* has not been reported to convert maltose into trehalose. Therefore, the microorganism isolated

by us can be recognized as a novel *Pseudomonas stutzeri* strain and has been designated as *Pseudomonas stutzeri* CJ38.

We constructed the restriction map of a recombinant plasmid pCJ104 of the present invention using various restriction enzymes. Two trehalose synthase gene sequences are known (Biochim. Biophys. Acta 1996, 1290, 1-3 and Biochim. Bophys. Acta 1997, 1334, 28-32). The comparison of the present and known restriction maps revealed that pCJ104 represents different patterns from those known.

Trehalose synthase proteins from known microorganisms have shown similarities in their N-terminus. However, it was found that the N-terminal sequence of the trehalose synthase protein of the present invention is not identical with those of known trehalose synthase proteins. The results are shown in Table 1 below.

Table 1. N-terminal Sequences of Trehalose Synthase Proteins

Source of Trehalose Synthase		N-terminal Sequence
Known Microbes	*Thermus aquaticus* ATCC 33923	M-D-P-L-W-Y-K-D-A-V-I-Y-Q-
	Pimelobacter sp. R48	S-T-V-L-G-E-E-P-E-W-F-R-T-A-V-F-Y-E-
	Pseudomonas putida H262	G-K-W-P-R-P-A-A-F-I-D-
Transformed *E. coli* of the Present Invention		S-I-P-D-N-T-Y-I-E-W-L-V-

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The nucleotide sequence of 4.7 kb *Sau*3AI fragment within a recombinant plasmid pCJ104 of the present invention and the amino acid sequence of a trehalose synthase protein expressed therefrom were determined (SEQ ID NO: 1).

In addition, the intact sequence of a trehalose synthase protein of the present invention was compared to those of the trehalose synthase proteins disclosed in Biochim. Biophys. Acta 1996, 1290, 1-3 and Biochim. Biophys. Acta 1997, 1334, 28-

32. The comparison revealed that there are no similarities between them.

The enzymatic conversion reaction was carried out using crushed E. coli transformants including recombinant plasmids pCJ104 or pCJ122. As a result, the titer of trehalose synthase enzyme from the crushed cells of the present invention was considerably higher than that from the wild type *Pseudomonas stutzeri* CJ38.

The properties and availabilities of the plasmids and microorganisms used in and prepared by the present invention are shown in Table 2 below.

Table 2

Microbes and Plasmids	Properties	Availability
Pseudomonas stutzeri CJ38	Wild type strain producing the trehalose synthase enzyme of the present invention	KFCC-10985
E. coli NM522	hsdΔ5, Δ(lac$^-$pro) [F', Pro$^+$, lacI^{q}ZΔM15]	Amersham
E. coli ATCC35467	[malP,Q::Tn5 ompBCS1 F$^-$ araD139Δ(argF$^-$ lac) 205U169 rpsL150 relA1 flbB5301 deoC1 ptsF25]	ATCC
pCJ104	pUC18 containing 4.7 kb Sau3AI DNA fragment (trehalose synthase gene), Ap^r	Constructed
pCJ121	pUC18 containing 3.35 kb KpnI DNA fragment (trehalose synthase gene), Ap^r	Constructed (Control)
pCJ122	pUC18 containing 2.5 kb BamHI-BglII DNA fragment (trehalose synthase gene), Ap^r	Constructed
pCJ123	pUC18 containing 1.2 kb BamHI-EcoRI DNA fragment	Constructed (Control)
pUC18 and pUC19	Ap^r, 2.7 kb	New England Biolabs

Nutrient medium (0.3% broth, 0.5% peptone, pH 6.8) and LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) were used for cultivation of *Pseudomonas stutzeri* and *E. coli*, respectively. For the culture of cells transformed by electroporation, SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was used. MacConkey agar medium (4% bacto MacConkey agar base, 2.0% maltose, pH 7.0) was used in cloning the trehalose synthase gene. Ampicillin was added in a concentration of 50 mg/L. Gene Pulser (Bio-Rad) was used in transformation of *E. coli* by electroporation. The genetic manipulation used in the present invention was carried out in accordance with procedures described in *Molecular Cloning, Laboratory Manual*, 2nd ed., Sambrook, J., E.F. Frishc and T. Maniatis and *Guide to Molecular Cloning Techniques, Methods in Enzymol.* Vol. 152, Berger, S.L., A.R. Kimme.

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The enzymatic reaction is conducted at pH 6.0 to 7.0, preferably pH 7.0 to 10, and at temperatures of 4°C to 45°C, preferably 20°C to 40°C. Maltose can be used as a substrate in a concentration of less than 50%. The trehalose synthase enzyme can be used in a pure form or in crushed cells.

The following examples illustrate the present invention. From the foregoing description and the following examples, it is believed that those skilled in the art would be able to carry out the invention completely.

Example 1
Screening of Microorganism

A platinum loop of microorganisms, isolated from soil, was inoculated in a 500 ml Erlenmeyer flask containing 50 ml of LB culture solution (0.5% of yeast extract, 1.0% of bactotrypton, 0.5% of salt) and cultured at 28°C for 2 days. The culture was centrifuged at 4°C, 8,000 rpm, for 5 minutes. The cells were collected and washed with physiological saline. The washed cells were suspended in 10 ml of phosphate buffer solution (10 mM, pH 7.0). The cells were crushed by an ultrasonicater and the crushed cells were centrifuged at 4°C, 1,200 rpm, for 20 minutes and the supernatant was used as a crude enzymatic solution. The

concentration of the protein in the crude enzymatic solution was determined by the Bredford method. 100 μg of protein was mixed with 20 μl of 100 mM maltose and 10 μl of 100 mM phosphate buffer solution (pH 7.0). Distilled water was added to the mixture until the total volume reached 100 μl and the reaction occurred at 30°C for 20 hours. The saccharides present in the reaction solution were analyzed by TLC, HPLC, and GC.

Example 2
Analysis of Trehalose by Thin-layer Chromatography (Figure 1)

After the reaction was completed, 5 μl of the reaction solution were spotted on Kieselgel 60 TLC (Merck, Germany) and placed in a vessel containing a solvent system of n-butanol-pyridine-water (7:3:1) to develop the specimens. It was sprayed with a solution of 20% sulfuric acid in methyl alcohol and dried at 110°C for 10 minutes. The saccharides in the specimens were thus specified. Among at least 1,000 soil microorganisms investigated, two were confirmed to have the ability to convert maltose into trehalose. Figure 1 shows that trehalose did not exist in the specimens prior to the reaction but, after completion of the reaction, saccharides were detected at the site of a standard trehalose specimen.

Example 3
Analysis of Trehalose by Gas Chromatography (Figure 2)

After completion of the reaction, 10 μl of the reaction solution was dried by a reduced pressure dryer. The dried product was dissolved in 20 μl of dimethylformamide and the resulting solution was mixed with the same volume of bis(trimethyl)trifluoracetamide to form trimethylsilane derivatives. One μl of aliquot was used in GC analysis. As shown in Figure 2, the peak of the reaction solution was observed to occur at the same time as with a standard trehalose specimen.

Example 4
Analysis of Trehalose by High Performance Liquid Chromatography (Figure 3)

After the reaction was completed, half of the reaction solution was mixed with the same volume of phenol to remove proteins. The specimen solution thus obtained was used in the HPLC analysis. The peak of the specimen was observed to occur at the same time as with a standard trehalose specimen. The remaining half of the reaction solution was heated to 100°C for 10 minutes to terminate enzyme activity. It was reacted at 37°C for 10 minutes with trehalase (Sigma) which specifically acts on α-1,1-trehalose. After completion of the reaction, the solution was mixed with the same volume of phenol solution to remove proteins. The solution obtained thus was subjected to HPLC, and as a result the peak disappeared at the same time as with a standard trehalose.

Example 5

Identification of Microorganism Capable of Converting Maltose into Trehalose

The soil microorganism of the present invention was observed by electron microscope and is characterized by rod shaped bacteria with flagellum. It was also characterized as aerobic by an O/F test and by Gram-negative. The physiological characteristics of the microorganism are summarized in Table 1. These characteristics of the present microorganism were compared to those of microorganisms described in *Bergy's Manual of Systemic Bacteriology*, 1984 and in patent publications, and it was classified as *Pseudomonas stutzeri*, because it is almost identical to that microorganism, physiologically and morphologically.

Table 1

DP3 -	OFG +	GC +	ACE -	ESC -	PLI -
URE -	CIT +	MAL +	TDA -	PXB -	LAC -
MLT +	MAN +	XYL -	RAF -	SOR -	SUC -
INO -	ADO -	COU -	H2S -	ONP -	RHA -
ARA -	GLU -	ARG -	LYS -	ORN -	OXI -
TLA -					

Example 6

Cloning of Trehalose Synthase Gene (Figure 4)

(1) Isolation of Chromosomal DNA from *Pseudomonas stutzeri*

Pseudomonas stutzeri was grown in a nutrient medium and at an early resting stage, cells were recovered by centrifugation. The recovered cells were washed twice with TE solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The washed cells were suspended in 20 mL of STE buffer (20% sucrose, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) and 5 mg/mL of lysozyme and RNase A were added to the suspension. The reaction occurred at 37°C for 2 hours. After the reaction was completed, SDS was added up to a concentration of 1% and the reaction continued at 37°C for 30 minutes. This solution was reacted with the same volume of phenol for 4 hours and was subjected to centrifugation. 5M NaCl was added to the resulting supernatant until its concentration reached 0.1 M. Using a glass bar, a two-fold volume of anhydrous ethanol was added to obtain chromosomal DNA. The chromosomal DNA was washed with 70% ethanol and dissolved in TE solution for use in the next experiment.

(2) Preparation of Genomic Library

The pure chromosomal DNAs isolated from *Pseudomonas stutzeri* were partially digested with restriction enzyme *Sau*3AI at 37°C for 15 to 30 minutes. The restriction enzyme was inactivated with heat and agarose gel electrophoresis was carried out to obtain 3 to 10 kb DNA fragments. As shown in Figure 5, plasmid pUC18 was digested with *Bam*HI and was treated with calf intestinal phosphatase. The cleaved DNAs were mixed with 3 to 10 kb DNA fragments previously obtained and ligation with T4 DNA ligase was allowed at 15°C for 16 hours. The recombinants thus obtained were used for transformation. The transformation was carried out by electroporation as follows. *E. coli* NM522 was cultured on LB medium for 14 to 15 hours. The resulting culture was inoculated on 1L LB so that initial absorbency became 0.07 to 0.1 at 600 nm, and then cultivation was allowed until the absorbency reached 0.8. The cells were centrifuged and suspended in 1L of HEPES [N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)] buffer solution. The cells were again centrifuged and suspended in 500 ml of cold sterile deionized distilled water.

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The cells were again centrifuged and suspended in 20 ml of 10% glycerol solution. The cells were again centrifuged and suspended in 2 to 3 ml of 10% glycerol solution so that the cell concentration was adjusted to 2-4 x 1,010/ml. The cell suspension was rapidly frozen and stored at -70°C. The frozen cells could be used for about one month during which time their transformation frequency did not decrease. 40 μL of frozen cell suspension was thawed in ice and the restored suspension was mixed with the ligated DNA solution. The mixture was put in a gene pulser cuvette with a diameter of 0.2 cm and the capacitance and strength of electric field was fixed at 25 uF and 12.5 kV/cm, respectively. After a single electric pulse was passed at resistance of 200 to 400 Ω, 1 ml of SOC medium was immediately added and cultured at 37°C for 1 hour. The culture was streaked on LB-ampicillin agar medium and cultivation was allowed for 24 hours to obtain at least fifty thousand colonies. These colonies were together cultured in LB broth for 2 hours. DNA was purely isolated using an alkaline lysis and the genomic library was constructed therefrom.

(3) Cloning of Trehalose Synthase Gene

E. coli ATCC35467, which is unable to utilize maltose as a carbon source, was transformed with the genomic library obtained from the above by electroporation. The transformed cells were streaked on a MacConkey-ampicillin agar medium containing 20 g/L of maltose. Once the trehalose synthase gene of *Pseudomonas stutzeri* is introduced into *E. coli*, maltose is converted into glucose by the trehalase present in E. coli. As the resulting glucose is metabolized, pH decreases and thereby the color of the colonies on the MacConkey agar medium changes from yellowish to red. This principle was applied to the present cloning system. The transformed *E. coli* ATCC35467 with the genomic library was cultured on a MacConkey agar medium to obtain red colonies. The isolation of plasmid DNA revealed that it contained about 4.7 kb DNA fragment. The plasmid was designated as pCJ104. To assay enzymes, *E. coli* ATCC35467/pUC18 (control), *E. coli* ATCC35467/pCJ104 and wild type *Pseudomonas stutzeri* CJ38 were cultured. *E. coli* cells were grown on a LB medium until their early resting stage. *Pseudomonas stutzeri* CJ38 was grown on a nutrient medium. The cells were separated by centrifugation and crushed. The crushed cells were reacted with 20% maltose as substrate in 20 mM diethanolamine as buffer

solution, at pH of 8.5 to 9.0 and a temperature of 35°C. 1.0% trichloroacetic acid was added to the reaction solution, which was then subjected to centrifugation and high performance liquid chromatography to assay the quantities of maltose and trehalose. The results are shown in Table 3 below.

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Table 3. Enzyme Titration

Microorganisms	Non-enzymatic activities (U*/mg of protein)	Culture Titer (U/ml of culture solution)
Pseudomonas stutzeri CJ38	0.1	0.023
E. coli ATCC35467/pUC18	0	0
E. coli ATCC35467/pCJ104	0.26	0.175

*U=μmol trehalose/minutes

Example 7

Restriction Map Construction of Trehalose Synthase Gene (Figure 5)

The plasmid pCJ104 was separated using conventional methods and treated with various restriction enzymes to construct a restriction map.

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The plasmid pCJ104 was subjected to single, double, and triple-digest procedures using about twenty restriction enzymes, such as *Aat*II, *Bam*HI, *Eco*RI, *Eco*RV, *Kpn*I, *Nco*I, *Nde*I, *Pst*I, *Sac*I, *Sac*II, *Sal*I, *Sph*I and *Xho*I. DNA fragments were analyzed by electrophoresis through agarose gel and compared to construct the restriction map.

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~~Example 3~~

Subcloning of Trehalose Synthase Gene and Enzyme Assay

(1) Subcloning of Trehalose Synthase Gene (Figure 6)

A subcloning was carried out to determine the sites of the trehalose synthase gene in 4.7 kb plasmid pCJ104. The plasmid pCJ104 was cleaved with *Kpn*I and a 3.35 kb fragment was isolated. This fragment was introduced into vector pUC18/*Kpn*I/CIP and *E. coli* NM522 was transformed with the resulting recombinant. The recombinant plasmid pCJ121 with a directional cloning of 3.35 kb fragment into pUC18/*Kpn*I was constructed. In addition, the plasmid pCJ104 was cleaved with double digestions of *Bam*HI and *Bgl*II. The 2.5 kb *Bam*HI-*Bgl*II fragment thus obtained was purified and ligated into pUC18/*Bam*HI/CIP, followed by transformation of *E. coli* NM522 with the recombinant. The recombinant plasmid pCJ122 with directional cloning of 2.5 kb *Bam*HI-*Bgl*II fragment into pUC18/*Bam*HI was constructed. Finally, the plasmid pCJ104 was double-digested with *Bam*HI and *Eco*RI and the resulting 1.2 kb *Bam*HI-*Eco*RI fragment was purified. This fragment was ligated into vector pUC18/*Bam*HI/*Eco*RI and *E. coli* NM522 was transformed with the recombinant. The recombinant plasmid pCJ123 was constructed.

E. coli ATCC35467 was transformed with each of the constructed recombinant plasmids. The transformants were cultured on a MacConkey-ampicilline agar medium containing 2.0% maltose (20 g/L) and the color of the colonies formed therefrom was observed. It was observed that the *E. coli* ATCC35467 carrying pCJ121 and pCJ122 formed red colonies but that the *E. coli* ATCC35467 carrying pCJ123 formed yellow colonies since it did not decompose maltose. Therefore, it can be seen that the trehalose synthase gene is located in the larger 2.5 kb *Bam*HI-*Bgl*II fragment, rather than in the 1.2 kb *Bam*HI-*Eco*RI fragment.

(2) Titration of Trehalose Synthase of Transformant Containing Subcloned Plasmid

Transformed *E. coli* ATCC35467/pCJ121, ATCC35467/pCJ122 and ATCC35467/pCJ123 were cultured on an LB-Ap medium until the early resting stage. The cells were recovered by centrifugation and washed twice with an appropriate volume of 20 mM diethanolamine solution. The washed cells were suspended in an

appropriate volume of 20 mM diethanolamine solution and crushed by ultrasonicator. The crushed cells were centrifuged and the supernatant obtained therefrom was used as enzymatic liquid. The supernatant was reacted with 20% maltose solution containing 20 mM diethanolamine, pH 8.5 to 9.0 at 35°C. 1.0% trichloroacetic acid was added to the reaction solution, and centrifugation and HPLC were conducted for analysis. One unit of enzyme activity was defined as a quantity of enzyme when it produced 1 μmol of trehalose per minute. The results are shown in Table 5 below.

According to the double titration, the enzyme titer of *E. coli* ATCC35467/pCJ122 was the highest. *E. coli* ATCC35467/pCJ122 was cultured in high density under the conditions described in Table 6 below in 5 L fermenter. As a result, the non-enzymatic activity was 5.0 U/mg of protein, equal to that obtained by culturing it on an LB medium, and the titer of the trehalose synthase enzyme in the high density culture was increased to 30 U/ml of culture (Table 5). The non-enzymatic activity and culture titer of *E. coli* ATCC35467/pCJ122 were increased 50 times and about 1,300 times, respectively, compared to wild type *Pseudomonas stutzeri*.

Table 5

Microorganisms	Non-enzymatic Activity (U/mg of protein)	Culture Titer of 5 L Fermenter (U/ml of culture)
E. coli ATCC35467/pCJ121	0.43	-
E. coli ATCC35467/pCJ122	4.95	30
E. coli ATCC35467/pCJ123	0	-

Table 6

Fermentation Medium	g/L	Culture Condition
glycerol	50	pH 7.0
$(NH_4)_2SO_4$	6	Temperature of 33°C
KH_2PO_4	2	800 rpm

$MgSO_4 \cdot 7H_2O$	1	1.0 vvm
Yeast Extract	5	
Trace Elements	1 ml	
Amino Acids (Threonine, Leucine, Isoleucine, Valine, Histidine, Arginine)	0.5	